Local lentiviral shRNA silencing of CCR2 inhibits vein graft thickening in hypercholesterolemic APOE*3Leiden mice

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Abstract

**Objective:** Inflammatory responses to vascular injury are key events in vein graft disease and accelerated atherosclerosis, which may result in bypass failure. The monocyte chemoattractant protein (MCP)-1/CC-chemokine receptor (CCR)-2 pathway is hypothesized to play a central role. Here, we study the effect of local application of lentiviral short hairpin RNA (shRNA), targeted against CCR2, in a murine model for vein graft disease.

**Methods and Results:** A venous interposition was placed into the carotid artery of hypercholesterolemic APOE*3Leiden mice to induce vein graft thickening with features of accelerated atherosclerosis. Vascular CCR2 and MCP-1 mRNA expression levels were significantly upregulated during lesion progression in the vein graft. Infection of smooth muscle cells with a lentiviral shRNA targeting murine CCR2 (shCCR2) completely abolished MCP-1 induced smooth muscle cell migration and inhibited smooth muscle cell proliferation *in vitro* (n=3 per group).

To demonstrate the efficacy of the lentiviral shCCR2 in blocking vein graft disease *in vivo*, lentiviral shCCR2 or a control lentivirus was used to infect the vein graft locally (n=8). Morphometric analysis on sections of grafts shows a significant 38% reduction in vein graft thickening in the shCCR2 treated animals, four weeks after surgery (control: 0.42±0.05 mm² and shCCR2: 0.26±0.03 mm²; P=0.007).

**Conclusion:** These data demonstrate that vascular CCR2 contributes to vein graft disease and that local application of shRNA against CCR2 to the vessel wall prevents vein graft thickening in hypercholesterolemic mice, suggesting that local overexpressing of shRNA using organ targeted lentiviral gene delivery may be a promising therapeutic tool to improve vein graft disease in bypassed patients.
Introduction

Venous bypass graft surgery is a general applied treatment for both peripheral and cardiac arterial occlusive disease. Failure of the graft is a serious clinical problem, with a primary patency of 61% after 1 year, as reported in the Project of Ex-vivo Vein graft Engineering via Transfection (PREVENT) III trial\(^1\), up to a patency of 40% ten years after bypass surgery, frequently resulting in myocardial infarction, the need of reinterventions or limb amputations\(^2\). The development of vein graft thickening, mainly caused by intimal hyperplasia and accelerated atherosclerosis, is triggered by inflammatory activation of vascular cells and is the major cause of late graft failure\(^3\).

Vein graft thickening starts with the adhesion and influx of monocytes into the intima of the vein graft. Additionally, vascular smooth muscle cells migrate and proliferate and macrophages accumulate in the vessel wall\(^4\). Subsequently, macrophages take up oxidized lipoproteins and develop into foam cells, especially in a hypercholesterolemic environment, consequently leading to accelerated atherosclerosis in the vein graft\(^5\).

Inflammation is thought to play a pivotal role in this process\(^6\). Monocyte chemoattractant protein (MCP)-1, one of the most potent chemotactic agents to monocytes, and its receptor CC-chemokine receptor (CCR)-2 are key mediators in vascular inflammation\(^7\). Recently, we demonstrated that vein graft thickening in a mouse in vivo model could be inhibited strongly by systemic overexpression of 7ND-MCP-1, a mutant form of MCP-1 that acts as a dominant antagonist of CCR2\(^8\).

Since MCP-1 is a key chemokine in inflammatory processes, prolonged systemic inhibition of MCP-1 may have negative side effects. Thus, systemic overexpression of inhibitors of the MCP-1/CCR2 pathway is not a preferred therapeutic treatment. Therefore, we designed the present study to interfere in the MCP-1/CCR2 pathway locally in vein grafts to prevent or reduce vein graft thickening.

In this study, a lentiviral vector expressing a short hairpin RNA (shRNA) construct, developed against CCR2 (shCCR2) was used. This vector was found to lead to effective silencing of CCR2 both, in vitro (in CCR2-transfected cells) and in vivo in a model of peritonitis\(^9\). Here, the effect of shCCR2 on MCP-1 induced smooth muscle cell migration and smooth muscle cell proliferation was described.

To assess the effect of shCCR2 in vivo, a venous interposition model in APOE*3Leiden mice on a hypercholesterolemic diet was used in this study. After placing a venous interposition in the carotid artery of these mice, vein
graft disease consisting of vessel wall thickening with signs of accelerated atherosclerosis develops within four weeks\textsuperscript{10,11}.

First, CCR2 and MCP-1 mRNA expression patterns in time were studied in this vein graft model and finally, we describe the effect of locally applied lentiviral shRNA against CCR2 on vein graft thickening in hypercholesterolemic APOE\textsuperscript{*3} Leiden mice.

**Methods**

**Mice and vein graft model**

Animal experiments were approved by the animal welfare committee of the Netherlands Organization for Applied Scientific Research (TNO). For all experiments, 12 week old male APOE\textsuperscript{*3} Leiden animals on a C57BL/6 background, bred at the TNO laboratory, were used. These mice develop diet dependent hypercholesterolemia (levels between 7.5-12.5 mmol/L) and atherosclerosis\textsuperscript{12}. All mice received water and food ad libitum. Animals were fed with a cholesterol-enriched high-fat diet, containing 1\% cholesterol and 0.05\% cholate (Arie Blok B.V.), starting 4 weeks before surgery and continued during the whole experiment\textsuperscript{11}. Cholesterol levels in serum were determined one day before surgery and at sacrifice, using a cholesterol esterase, cholesterol oxidase reaction (Chol R1; Roche Diagnostics). Prior to surgery and sacrifice, mice were anaesthetized by an intraperitoneal injection with a combination of Midazolam (5 mg/kg, Roche), Medetomidine (0.5 mg/kg, Orion) and Fentanyl (0.05 mg/kg, Janssen-Cilag). Mice with a mean weight of 26.7±0.5 grams were allocated randomly to the different experimental groups (n=8 per group for the shCCR2 experiment and n=3 per time point for the immunohistochemistry and cytokine expression time courses).

For the vein graft model, a venous interposition was placed in the carotid artery as described previously\textsuperscript{10}. In brief, caval veins of donor mice serving as vein grafts were harvested and preserved in 0.9\% NaCl containing 100 IU heparin. In the recipient mice, the right carotid artery was dissected free from its surroundings and cut midway. Next, a polyethylene cuff was placed at the end on both sides. The artery was everted around the cuff and ligated at both ends. Finally, the caval vein was grafted by sleeving the ends of the vein over two everted ends of the carotid artery and ligating them together.
Vein graft thickening quantification and immunohistochemistry

At sacrifice, tissue segments were harvested after in vivo perfusion fixation with PBS/formaldehyde 4% and paraffin-embedded. To quantify vein graft thickening, sequential cross-sections were made throughout the embedded vein grafts. For each mouse, six equally spaced, representative sections per vessel segment (n=8 per group) were used after being stained for haematoxylin-phloxine-saffron (HPS).

Vein graft thickening was defined as the area between lumen and adventitia and determined by subtracting the luminal area from the total vessel wall area. No distinction between intima and media was made, since there is no morphological border between the neointima and media (like internal elastic lamina in arteries)\(^\text{13}\). Image analysis software was used to quantify the vein graft thickening (Leica QWin).

The composition of vein graft thickening was visualized by HPS staining and immunohistochemistry (n=8 per group). Smooth muscle cells and macrophage-derived foam cells were visualized with α-smooth muscle cell actin staining (anti-SM α-actin, dilution 1:750, Roche Applied Biosciences) and AIA31240 antibody (1:1000, Accurate Chemical), respectively. MCP-1 and CCR2 expression in the vein graft wall were detected by using an anti-mouse JE/MCP-1 antibody (1:20, BD Biosciences) and an anti-CCR2 antibody (1:200, Abcam).

Smooth muscle cells and macrophage-derived foam cells immunopositive areas in the vein graft thickening were calculated as a percentage of the total vein graft thickening area in cross sections by means of image analysis software (Leica QWin). CCR2 expression was quantified by counting the CCR2-positive cells in the vessel wall per slide and divided by total number of nuclei in these cross-sections as determined by haematoxylin staining. All histological quantification was performed in a single blinded fashion.

RNA isolation and Real-Time quantitative RT-PCR gene expression analysis

For gene expression analysis, vein grafts were harvested and directly snap-frozen in liquid nitrogen at several time points (6 hours, 1, 3, 7, 14 and 28 days after surgery; n=3 per time point). RNA was isolated, using RNasy Fibrous Tissue Mini Kit and DNA contaminants removed by treatment with RNase Free DNase set according to manufacturer’s protocols (Qiagen). cDNA was synthesized by means of Ready-To-Go Beads (Amersham Biosciences).
Quantitative analysis of mRNA levels was performed using an ABI Prism 7700 sequence detection system (Applied Biosystems) as described previously and intron-spanning PCR primer sets were designed using Primer Express 1.5 software (Table 6.1; Applied Biosystems). Relative mRNA expression levels ($\Delta C_t$) were calculated by subtracting the average cycle threshold ($C_t$) per time point from the averaged $C_t$ values of two housekeeping genes: hypoxanthine phosphoribosyltransferase (HPRT) and cyclophilin ($\Delta C_t = C_{t \text{target gene}} - C_{t \text{average housekeeping genes}}$). The $\Delta C_t$ levels at each time point were compared to the mRNA expression levels of CCR2 and MCP-1 before surgery ($\Delta \Delta C_t$) and a mean fold induction was calculated as $2^{-\Delta \Delta C_t}$.

Table 6.1. Primer sequences used for Real-Time RT-PCR analysis

<table>
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<th>Gene</th>
<th>Forward (5’-3’)</th>
<th>Reverse (5’-3’)</th>
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<td>Cyclophilin</td>
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<td>ATTTTGTCTTAACACTGTTGTT</td>
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<td>mHPRT</td>
<td>TGCTCGAGATGTCAAGGGA</td>
<td>AGCAGGTCAGCAAGAAGACTTATAG</td>
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<td>MCP-1</td>
<td>GCATCTGCCTAAGGTCCTTA</td>
<td>TTCACTGTCAACAGGTCACACCTTA</td>
</tr>
<tr>
<td>CCR2</td>
<td>AACTGTGTGATTGACAAGCACCCTAGAC</td>
<td>TGACAGGATTAATGCGACAGTTG</td>
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Design, cloning and validation of shRNA directed against murine CCR2

Short hairpin CCR2 and lentiviral vectors, as described by Bot et al., were used to intervene in the vein graft model. Briefly, a pair of 64-nucleotide oligonucleotides encoding a 19-nucleotide CCR2 shRNA (5’-GATCCCCCTGTGTGATTTGACAAGCAC TTCAAGAGA GTGCTTGTCATACTCACA-CAG TTTTTGGAAA-3’ and 5’-AGCTTTTCCAAAA ATGTGTGATT-GACAAGCAC TCTCTTGGAA GTGCTTGTCATACTCACAG GGG-3’) were designed. Nucleotides 867 to 885 (underlined) represent the sequence targeting murine CCR2. The pSUPER-H1.shCCR2 vector was constructed by annealing and cloning the 64-nucleotide oligonucleotides into the pSUPER vector. Subsequently, lentiviral expression vectors (Lenti-shCCR2 and control virus) were constructed, produced and validated as previously described.
Viral transduction of vein grafts in vivo

Viral transduction of vein grafts was performed by application of lentiviral constructs dissolved in pluronic gel\textsuperscript{15}. During surgery, 100 µL pluronic-127 gel (20% wt/vol; Sigma Diagnostics) dissolved in 0.9% NaCl containing 10\textsuperscript{8} IU Lenti-shCCR2 or a control lentiviral vector expressing green fluorescent protein (Lenti-GFP) and 10 µg/mL diethylaminoethyl dextran (DEAE; Biotech AB) was applied to the adventitia. This formula allowed the solution to form a gel after contact with the vessel at 37 °C, generating a translucent layer that enveloped the grafted vessel segment and has the advantage of prolonged release of approximately 2 days\textsuperscript{16}. The wounds were closed after application of the gel.

Cell culture, smooth muscle cell migration and proliferation assay

Smooth muscle cells were obtained from thoracic aortas from male C57BL/6 mice using the collagenase digestion method\textsuperscript{17}. Cells were cultured in a humidified atmosphere (5% CO\textsubscript{2}) at 37°C in DMEM (Gibco, Invitrogen) containing 10% fetal calf serum (Gibco), 2 mmol/l l-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin (all from PAA).

Smooth muscle cells were infected with either Lenti-shCCR2 or control lentivirus at a multiplicity of infection (m.o.i.) of 15 in the presence of 10 µg/mL DEAE-dextran (3 wells per group). At two days after infection, smooth muscle cells were detached by short incubation with trypsin-EDTA (PAA) and seeded at a density of 5000 cells per well at an 80° angle as described previously to determine smooth muscle cell migration\textsuperscript{18}. After attachment of the cells to one side of the well, murine MCP-1 (100 ng/mL, Immunosource) was added, plates were repositioned at a 20° angle and the cells were incubated overnight, after which the number of cells that had migrated was scored manually and is expressed as a percentage of the control.

To determine the proliferative capacity of the smooth muscle cells expressing Lenti-shCCR2, cells were seeded at a density of 2\textsuperscript{*}10\textsuperscript{4} cells per well (n=3). The cells were allowed to attach and starved in DMEM containing 1% calf serum for 8 hours to synchronize the cell cycle. Subsequently, the cells were incubated for 24 hours with medium. After incubation, 0.5 µCi Methyl-3H-thymidine (Amersham Biosciences) was added per well and the cells were incubated overnight at 37°C. Hereafter, cells were lysated and [\textsuperscript{3}H] Thymidine incorporation was quantified as a measure for DNA synthesis in a liquid scintillation analyzer (Packard Tricarb 2900 TR, PerkinElmer) and is expressed in disintegrations per minute (counts/minute).
Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Statistical significance was calculated in SPSS 11.5 for Windows. Differences between groups were determined and expression data (ΔCt) were statistically analyzed using the Student’s T-Tests. Probability values of less than 0.05 were considered statistically significant.

Results

CCR2 and MCP-1 mRNA and protein expression profiles in murine vein grafts in vivo

mRNA expression of CCR2 and MCP-1 in vein grafts was examined during the development of vein graft thickening and accelerated atherosclerosis by real-time RT-PCR analysis (n=3 per time point). In untreated caval veins, expression of both, MCP-1 and its receptor CCR2, was minimal. Already six hours after engraftment, MCP-1 mRNA expression peaked with a 188±19-fold increase, expressed in fold induction as compared to mRNA expression level of MCP-1 in an untreated caval vein (P<0.001) and declined to a fold induction of 8.1±2.0 after 28 days (P=0.019); Figure 6.1A). In contrast to MCP-1, relative mRNA expression of CCR2 increased more gradually from a 2.1±0.2-fold induction, six hours after surgery (P=0.001; as compared to the untreated caval vein) towards an 11.0±2.3-fold upregulation after 28 days (P=0.009; Figure 6.1B).

Figure 6.1. Relative CCR2 and MCP-1 cytokine expression profiles in vivo.

Relative mRNA expression levels of MCP-1 (panel A) and CCR2 (panel B) in the vein graft at several time points after surgery, as determined by real-time RT-PCR (n=3 per time point). Relative mRNA expression is expressed as fold induction ± SEM, whereas the expression of MCP-1 or CCR2 mRNA in the normalized untreated veins is defined as “1”.

Panel B

Panel A
The *in vivo* presence of MCP-1 and CCR2 protein in murine vein grafts in time was demonstrated by immunohistochemistry (n=8 per group). In agreement with our previous findings, MCP-1 was mainly expressed by endothelial cells and in the adhering leukocytes in the first days after engraftment. At one and two weeks after surgery, MCP-1 was mostly detectable in the thickened vessel wall, colocalizing with AIA31240-positive monocytes/macrophages, as assessed with immunohistochemistry on serial sections and evaluating MCP-1 expression in relation to cell morphology. This implies that infiltrating macrophages express MCP-1\(^8\). Consistent with the mRNA expression of MCP-1, after an initial increase, the expression of MCP-1 protein in the vessel wall decreased in time and almost disappeared after four weeks (Figure 6.2A).

**Figure 6.2.** Immunohistochemical detection of MCP-1 and CCR2 in vein grafts in time.

Representative cross-sections of vein grafts harvested at 6 hours, 1, 14 and 28 days after surgery. MCP-1 (panel A) and CCR2 (panel B) expression in the vessel wall were visualized with an anti-mouse JE/MCP-1 and an anti-CCR2 antibody, respectively. MCP-1 and CCR2 expression could be detected in the adhering monocytes and in the infiltrating cells, depending on the stage of the developing vein graft thickening. Symbols indicate the several cell types (* for adhering leukocytes, # for macrophages and arrows for smooth muscle cells) and inserts represent enlargements of the specific cell types. Magnification of all images 200-400x. Scale bars represent 10 or 20 µM as indicated.

The expression pattern of CCR2 protein was comparable with MCP-1. CCR2 was present in the adhering leukocytes directly after surgery and expression increased throughout the whole vessel wall, colocalizing with infiltrating macrophages and smooth muscle cells, in the consecutive weeks (also assessed with immunohistochemistry on serial sections in relation to cell morphology; Figure 6.2B). Contrary to the MCP-1 expression, CCR2 protein expression did
not decline and was still detectable four weeks after bypass placement, which corresponds with the CCR2 mRNA expression data.

**Figure 6.3.** shCCR2 attenuates smooth muscle cell (SMC) migration and proliferation.

Panel A: Adding MCP-1 to cultured smooth muscle cells did not stimulate migration of smooth muscle cells which were transduced with lentiviral shCCR2, whereas migration of smooth muscle cells transduced with control vector was increased by adding MCP-1, as detected with a smooth muscle cell migration assay. Migration of cells was scored manually in one microscopic view and is expressed as a percentage of the control.

Panel B: Smooth muscle cell proliferation was inhibited in smooth muscle cells expressing lentiviral shCCR2 as compared to either smooth muscle cells transduced with Lenti-Empty or the control group. Proliferation capacity of smooth muscle cells was determined by means of quantifying [3H]thymidine incorporation as a measure for DNA synthesis. [3H] thymidine incorporation was measured in a liquid scintillation analyzer and is expressed in disintegrations per minute (counts/minute; error bars: mean ± SEM).

Lentiviral shCCR2 reduces vascular smooth muscle cell migration and proliferation

The functional effects of shCCR2 on smooth muscle cell migration and proliferation *in vitro* were studied in cultured smooth muscle cells, obtained from murine thoracic aortas. Smooth muscle cells were transduced with either Lenti-Empty as a control vector or Lenti-shCCR2 (3 wells with 5000 cells each per group). One day after stimulation with murine MCP-1, migration of smooth muscle cells infected with control virus was increased to 130±9% as compared to the non-stimulated control group. In contrast, adding MCP-1 did not stimulate migration of smooth muscle cells after being transduced with Lenti-shCCR2 (93±7% migration as compared to the control, P<0.001; Figure 6.3A).

Next, [3H]thymidine incorporation was used to assess smooth muscle cell proliferation. After transducing smooth muscle cells with either Lenti-Empty
or Lenti-shCCR2 and synchronizing the cell cycle, methyl-3H-thymidine was added (n=3). When transduced with Lenti-shCCR2, DNA synthesis was significantly decreased as compared to both, non-transduced smooth muscle cells and smooth muscle cells infected with the empty vector (Control: $85\times 10^4 \pm 1\times 10^4$ desintegrations per minute (dpm); Empty: $85\times 10^4 \pm 0.8\times 10^4$ dpm; shCCR2: $81\times 10^4 \pm 0.9\times 10^4$ dpm, P=0.031 and P=0.015 respectively, Figure 6.3B).

Local knock-down of CCR2 expression inhibits vein graft thickening in vivo

Before the effect of Lenti-shCCR2 on vein graft thickening could be studied, the transduction efficiency of lentiviral vectors to murine veins was determined (n=4 per group, Figure 6.4, Panel E).

To study the effect of shCCR2 on vein graft thickening in hypercholesterolemic mice, caval veins from donor mice were placed in male APOE*3Leiden mice. During surgery, vein grafts were transduced by application of lentivirus encoding for either Lenti-shCCR2 or Lenti-GFP as a control dissolved in pluronic gel (n=8 per group).

At the start of the experiment, mean serum cholesterol levels in the shCCR2 treated group were $11.1 \pm 3.0$ mmol/L and $7.6 \pm 1.0$ mmol/L in the control mice (difference not significant, p=0.13). Also during the experiment, mean cholesterol levels and body weights of all mice in both groups did not change significantly (data not shown).

Four weeks after bypass placement, histomorphometric analysis was performed on the vein grafts. A 38% reduction of vein graft thickening area was found in the Lenti-shCCR2 treated animals as compared to the control group (control: $0.42 \pm 0.05$ mm² and shCCR2: $0.26 \pm 0.03$ mm²; P=0.007; Figure 6.4, panel A). The total vessel wall area in the shCCR2 treated group was less expanded as compared to the control animals (control: $0.93 \pm 0.05$ mm² and shCCR2: $0.70 \pm 0.06$ mm²; P=0.006). However, luminal area did not diverge between both groups, indicating an inhibition of outward remodeling after shCCR2 treatment (control: $0.51 \pm 0.05$ mm² and shCCR2: $0.46 \pm 0.04$ mm²; P=0.26; Figure 6.4, panel B and C).

The effect of shCCR2 treatment on cellular composition of the vein graft lesions was studied by immunohistochemical analysis for the presence of smooth muscle cells and lipid-loaded macrophages (Table 6.2). Four weeks after surgery, no significant differences in relative contribution to the cellular composition were seen for both, the SM-actin positive area of the thickened vessel wall (expressed as positive stained area as a percentage of the total area)
when compared with the control group (control: 10±3%, shCCR2: 11±6%, P=0.45) as well as for the lipid-loaded macrophage content of the shCCR2 treated animals (control: 9±2% and shCCR2: 11±2%, P=0.22).

Although local application of shCCR2 resulted in a significant inhibition of vein graft thickening and thus reduced the absolute number of CCR2 positive cells in the vein graft lesions, this treatment did not alter the relative abundance of CCR2-positively stained cells in the vessel wall in both groups, after 28 days (control: 44±5%, shCCR2: 50±8%, P=0.52; Table 6.2).

Table 6.2. Quantitative analysis of effect of shCCR2 treatment on cellular plaque composition, remodeling and CCR2 content of vein grafts, 4 weeks after surgery

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<thead>
<tr>
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<th>Control</th>
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<tr>
<td>SMCs *</td>
<td>10.4±2.7</td>
<td>11.1±5.7</td>
</tr>
<tr>
<td>Macrophages *</td>
<td>9.0±1.5</td>
<td>10.8±1.8</td>
</tr>
<tr>
<td>CCR2 †</td>
<td>44.3±5.4</td>
<td>50.3±7.6</td>
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Results were determined by immunohistochemistry and values are expressed as mean ± SEM. All differences between both groups are not significant.

* Expressed as percentage of total vein graft thickening.
† Positively stained cells are expressed as a percentage of total number of cells.

Figure 6.4. Effect of shCCR2 on vein graft thickening in hypercholesterolemic APOE*3Leiden mice.

Vein graft thickening, total vessel wall and luminal area of APOE*3Leiden mice (n=8 per group) four weeks after vein graft surgery and local infection of the vein graft with lentiviral shCCR2 or a control lentivirus. Areas were quantified by using 6 sequential sections per segment and are expressed in millimetres squared (mean ± SEM).
Treatment with shCCR2 resulted in a significant reduction of vein graft thickening and total vessel wall area (Panel A and B; \(P = 0.007\)). Luminal area was not affected by shCCR2 treatment (Panel C). Panel D represents haematoxylin–phloxine–saffron (HPS) staining of vein grafts (vein graft thickening is indicated by black line, magnification 200x). Panel E represents fluorescence and light microscopy images of cryo-cross-sections of vein grafts after engraftment and infection with a lentiviral vector expressing green fluorescent protein (Lenti-GFP). Explanted vein grafts of donor mice were incubated in a solution of pluronic gel dissolved in 0.9% NaCl containing 108 IU Lenti-GFP or no virus to assess autofluorescence and 10 \(\mu\)g/mL DEAE dextran for 30 minutes at room temperature. Directly after implantation of these vein grafts in male APOE*3Leiden mice, this solution was applied to the adventitia where it, in an environment of 37°C, forms a translucent solid layer and envelops the graft. Three days after surgery, veins were harvested and cryo-cross-sections of the veins were made. GFP is expressed throughout the whole vessel wall after infection, whereas in the untreated veins only some auto-fluorescence is seen. The images were taken with a Leica DM-RE microscope (magnification 200x, scale bars represent 10 \(\mu\)M).
Discussion

Long-term patency of vein grafts is mainly compromised by vein graft thickening due to intimal hyperplasia and accelerated atherosclerosis. Inflammatory processes, initiated by damage of the vascular wall after surgery and an altered shear stress, lead to recruitment, adhesion and influx of monocytes, followed by smooth muscle cell migration and proliferation\(^6\). MCP-1 and its receptor, CCR2, are important mediators in monocyte recruitment and macrophage accumulation, thereby playing a key role in the process of vascular inflammation and (the initiation of) atherosclerosis\(^20\).\(^22\). We previously demonstrated the involvement of MCP-1/CCR2 pathway in the development of vein graft disease\(^8\).\(^23\). In this study, it is demonstrated that silencing of CCR2 in vascular cells by means of locally expressed shRNA reduces vein graft thickening in vivo, indicating new therapeutic interventions based on this approach.

The presence of MCP-1/CCR2 mRNA and protein during the development of vein graft thickening in vivo was demonstrated by real-time RT-PCR and immunohistochemistry, respectively. Both, MCP-1 mRNA and protein expression peaked early and diminished in time. CCR2 mRNA expression could be detected directly after surgery and increased gradually during vein graft development and is mainly transcriptionally regulated\(^24\), making it an attractive candidate for silencing via siRNA.

Next, targeted gene silencing by means of a lentiviral vector expressing shRNA, developed against CCR2 (shCCR2), was used to interfere in the MCP-1/CCR2 pathway. We show an inhibitory effect of Lenti-shCCR2 on MCP-1 induced smooth muscle cell migration and proliferation in vitro, which underscores the importance of MCP-1/CCR2 pathway in smooth muscle cells migration and proliferation next to its monocyte chemoattractant characteristics\(^8\) as well as on vein graft thickening in vivo.

RNA interference is a promising strategy to analyze gene function and to interfere in different disease models and may have a strong therapeutic potential. Because of the superior stability of siRNAs and an extended choice in target genes, the use of siRNAs is preferable to, for example, antisense oligodeoxynucleotides\(^25\). Application of RNAi in vivo requires efficient delivery of siRNA to the target tissue and long-term gene silencing\(^26\). Lentiviral vectors can transduct the vessel wall efficiently and warrant long-term shRNA expression as indicated in this study\(^27\).\(^28\). Moreover, third-generation lentiviruses, as used in this study, are self-inactivating and non-integrating
vectors that provide safe and stable transgene expression. When transduction is applied directly on the target tissue, the occurrence of systemic side effects is even less likely.

In the present study, gene specific silencing locally in the vascular target tissue was obtained by efficient and prolonged expression of a shRNA construct after lentiviral infection of the vein segments, resulting in inhibition of vein graft thickening in a murine model for vein graft disease.

The role of CC-chemokines in vein graft thickening was studied previously. Ali et al. reported that systemic adenoviral delivered broad-spectrum CC-chemokine inhibitor reduced accelerated atherosclerosis in vein grafts, underscoring the role of CC-chemokines in the development of vein graft disease. However, no conclusion about the role of CCR2 in particular could be drawn, due to the broad spectrum character of the CC-chemokine inhibitor used in this study. Alternatively, the use of siRNA or shRNA or specific receptor antagonists makes it possible to investigate a specific CC-chemokine or pathway. Recently, Tatewaki and colleagues described that local ex vivo adenoviral mediated gene transfer of CCR2 antagonist, 7ND-MCP-1, limited vein graft thickening in dogs. This is similar to our previous findings in mice, where we used systemic overexpression of a plasmid vector encoding the same antagonist (7ND-MCP-1) by means of electroporation mediated gene transfer into skeletal muscle and emphasizes the important role of MCP-1 and CCR2 in the pathogenesis of vein graft disease. However, the current generation adenovirus is not very suitable for an eventual clinical application, considering the relatively low transduction efficiency for vascular tissue, their preexisting immunity and the risk of undesired effects like liver toxicity. Although much progress is made in the generation of improved adenoviral vectors regarding vascular tissue infection, reduced immunogenicity and even with less pre-existing immunity in the human population, these newer generations of adenoviruses still are not fully applicable to the human situation.

In our study, shCCR2 has an inhibitory effect on smooth muscle cell proliferation and migration as well as on monocyte infiltration. This is reflected in an equal relative presence of smooth muscle cells and monocytes in the plaques of the vein grafts locally treated with shCCR2 and the control groups, although these plaques shows significant differences in size. Relative abundance of CCR2-positively stained cells in the vessel wall was not significant different in both groups after shCCR2 treatment. However, together with the reduction of the absolute number of CCR2 positive cells in the vein graft lesions, also the total number of vascular cells was reduced in this group due to inhibition
of vein graft thickening. Taken together, this indicates that shCCR2 not only hampered monocyte chemotaxis, but also had a direct inhibitory effect on smooth muscle cell migration and proliferation \textit{in vitro} as well as \textit{in vivo}.

In conclusion, in the present study we demonstrate that lentiviral delivery of shCCR2 blocks migration and proliferation of smooth muscle cells and inhibits vein graft thickening \textit{in vivo} when delivered directly and locally to the vessel wall, stressing the significant involvement of the MCP-1/CCR2 pathway in the process of vein graft disease in hypercholesterolemic APOE*3Leiden mice. Therefore, this local overexpression of shRNA by, for instance, lentiviral vector delivery as shown in this study, is a very promising therapeutic tool to improve vein graft patency.

\textbf{Acknowledgements}

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